

Peptide Antagonists of the Human Estrogen Receptor

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Estrogen receptor α transcriptional activity is regulated by distinct conformational states that are the result of ligand binding. Phage display was used to identify peptides that interact specifically with either estradiol- or tamoxifen-activated estrogen receptor α . When these peptides were coexpressed with estrogen receptor α in cells, they functioned as ligand-specific antagonists, indicating that estradiol-agonist and tamoxifen-partial agonist activities do not occur by the same mechanism. The ability to regulate estrogen receptor α transcriptional activity by targeting sites outside of the ligand-binding pocket has implications for the development of estrogen receptor α antagonists for the treatment of tamoxifen-refractory breast cancers.

About 50% of all breast cancers express the estrogen receptor α (ER α) protein and recognize estrogen as a mitogen (1). In a subpopulation of these tumors, antiestrogens, compounds that bind ER and block estrogen action, effectively inhibit cell growth. In this regard, the antiestrogen tamoxifen has been widely used to treat ER-positive breast cancers (2). Although antiestrogen therapy is initially successful, most tumors become refractory to the antiproliferative effects of tamoxifen within 2 to 5 years. The mechanism by which resistance occurs is controversial; however, it does not appear to result as a consequence of ER mutations or altered drug metabolism (3). It may relate instead to the observation that tamoxifen is a selective estrogen receptor modulator (SERM), functioning as an ER agonist in some cells and as an antagonist in others (4). Consequently, the ability of tumors to switch from recognizing tamoxifen as an antagonist to recognizing it as an agonist has emerged as the most likely cause of resistance. Upon binding ER, both estradiol and tamoxifen induce distinct conformational changes within the ligand-binding domain (5). The tamoxifen-induced conformational change may expose surfaces on the receptor that allow it to engage the general transcription machinery. We used phage display to identify specific peptides that interacted with the estradiol- and tamoxifen-ER complexes and used these peptides to show that estradiol and tamoxifen manifest agonist activity by different mechanisms.

Affinity selection of phage-displayed pep-

tide libraries was performed to identify peptides that could interact specifically with the agonist [17 β -estradiol (estradiol) or 4-OH tamoxifen (tamoxifen)], activated ER α , or ER β (6). Representative peptides from each of four classes presented in this study are shown in Fig. 1A. Several peptides that were isolated with estradiol-activated ER α (represented by α/β I) contained the Leu-X-X-Leu-Leu motif found in nuclear receptor coactivators (7). α II was isolated with either estradiol- or tamoxifen-activated ER α . Two classes of peptides, α/β III and α/β V, that interact specifically with tamoxifen-activated ER α and ER β , respectively, were identified. The

α/β V peptide was subsequently shown to interact with tamoxifen-activated ER α (6). Several additional peptides homologous to α/β V were identified. A BLAST search of the National Center for Biotechnology Information database with the derived consensus of the α/β V peptide class revealed that the yeast protein RSP5 and its human homolog, receptor potentiating factor (RPF1), both contain sequences homologous to α/β V. These proteins were previously shown to be coactivators of progesterone receptor B (PRB) transcriptional activity (8).

Peptide-peptide competition studies were performed with time-resolved fluorescence (TRF) to determine if the α II, α/β III, and α/β V peptides were binding the same or distinct "pockets" on the tamoxifen-ER α complex (9). The α/β III and α/β V peptides cross compete, and at equimolar peptide concentrations, 50% inhibition is observed (Fig. 1B). This result indicates that these two peptides bind to the same or overlapping sites on ER α . We believe that the α II peptide binds to a unique site as its binding was not competed by α/β V and only 50% inhibited by a 10-fold excess of the α/β III peptide.

We next assessed whether the peptides interacted with ER α in vivo using the mammalian two-hybrid system (10). The α/β I peptide interacted with ER α in the presence of the agonist estradiol but not the SERMs tamoxifen, raloxifene, GW7604, idoxifene, and nafoxidine or the pure antagonist ICI 182,780 (Fig. 2). The failure of antiestrogen-activated ER α to interact with the α/β I peptide is consistent with previ-

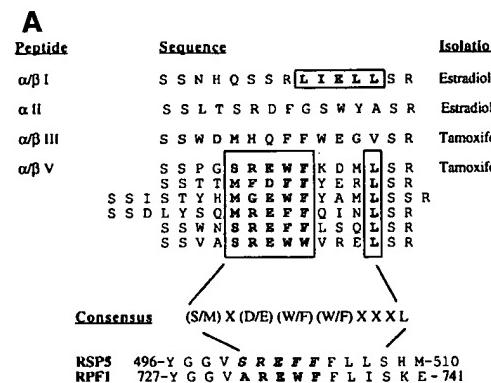
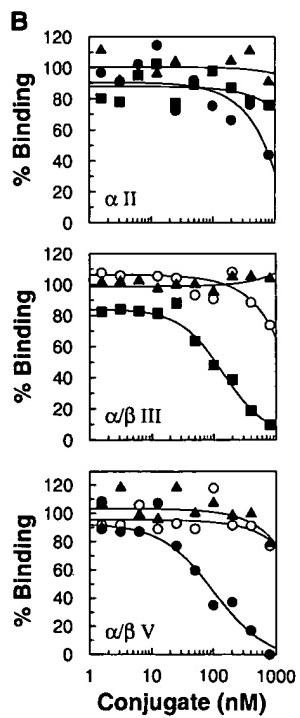


Fig. 1. Isolation of ER α -interacting peptides. (A) ER α -interacting peptides were isolated by phage display (6). Eighteen libraries were screened, each containing a complexity of about 1.5×10^9 phage. Several Leu-X-X-Leu-Leu (boxed)-containing peptides were isolated, of which α/β I is shown. One peptide each was isolated for the α II and α/β III peptide classes. Six peptides were isolated, including α/β V, that contained a conserved motif (boxed). Two proteins, RSP5 and RPF1, containing sequence homology to α/β V are shown. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, any amino acid; and Y, Tyr.



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ous studies that predict that the molecular mechanism of antagonism results from a structural change in the receptor ligand-binding domain that prevents coactivators from binding (5). α II interacted with the receptor in the presence of all modulators tested, with the unliganded (vehicle) and ICI 182,780-bound receptors showing the least binding activity. α / β III and α / β V interacted almost exclusively with the tamoxifen-bound ER α . ER α did not interact with the Gal4 DNA-binding domain (DBD) alone in the presence of any modulators tested. Further studies indicated that binding of α II, α / β III, and α / β V occurs within the hormone-binding domain between amino acids 282 and 535 (11) and, unlike binding of α / β I, does not require a functional activation function 2 (AF-2) (www.sciencemag.org/feature/data/1039590.shtml). These data indicate that SERMs induce different conformational changes in ER α within the cell and firmly establish a relation between the structure of an ER α -ligand complex and function.

When we examined the specificity of interaction between the peptides and heterologous nuclear receptors, we found, as expected, that the α / β I peptide interacted with ER β , PRB, and the glucocorticoid receptor (GR) when bound by the agonists estradiol, progesterone, and dexamethasone, respectively (Fig. 3, A, B, and C). The α / β V peptide interacted with tamoxifen-bound ER β and unexpectedly with PRB in the presence of the antagonists RU 486 or ZK 98299 (Fig. 3, A and B). The α / β V peptide, however, did not interact with the GR when bound by RU 486 or ZK 98299. α II and α / β III peptides failed to interact with ER β , PRB, or GR.

We next tested the ability of the peptide-Gal4 fusion proteins to inhibit ER α transcriptional activity. Tamoxifen displayed partial agonist activity when analyzed with the ER-responsive complement 3 (C3) promoter in HepG2 cells (Fig. 4A). This activity can reach 35% of that exhibited by estrogen and is mediated by three nonconsensus estrogen response

elements (EREs) located in the C3 promoter (12). When expressed in this system, the α / β I and α II peptides inhibited the ability of estradiol to activate transcription up to 50% and 30%, respectively (Fig. 4B). Two copies of the Leu-X-X-Leu sequence found in α / β I enhanced the inhibitory effect of this peptide and blocked estradiol-mediated transcription by about 90% (13). The inability of α / β III and α / β V to block estradiol-mediated transcription correlates well with their inability to bind the receptor when bound by agonist. Expression of α II, α / β III, and α / β V peptides blocked the partial agonist activity of tamoxifen (Fig. 4C). α II and α / β V were the most efficient disrupters of tamoxifen-mediated transcription, inhibiting this activity by about 90%. All peptide-Gal4 fusion proteins were expressed at similar levels, indicating that the relative differences in inhibition are not due to peptide stability (11). We also demonstrated that receptor stability and DNA binding are not affected by peptide expression (11). As expected, α / β I was unable to inhibit tamoxifen-mediated transcription. These findings are in agreement with the binding characteristics of these peptides and suggest that the pocket or pockets recognized by α II, α / β III, and α / β V are required for tamoxifen partial agonist activity. Although α / β V was shown to interact with PRB when bound by RU 486 (Fig. 3B), it was unable to block the partial agonist activity mediated by PRB/RU 486 (11). This result suggests that ER α /tamoxifen and PRB/RU 486 partial agonist activities are manifested differently. However, because α / β V was selected against ER α , this peptide may not bind PRB with high enough affinity to permit it to be useful as a PRB peptide antagonist.

Finally, we examined the ability of these peptides to inhibit ER transcriptional activity mediated through AP-1-responsive genes. This pathway has been proposed to account for some of the cell-specific agonist activity of tamoxifen (14). Both estradiol and tamoxifen activated transcription from the AP-1-responsive collagenase reporter gene, pCOL-Luc (Fig. 4D).

This activity is manifest in the absence of an ERE and is believed to occur through a mechanism involving an interaction between ER α and the promoter-bound AP-1 complex (14). Regardless of the mechanism, each peptide was able to inhibit ER α -mediated transcriptional activity in a manner that reflected its ability to interact with the receptor in a ligand-dependent manner (Fig. 4E).

The mechanism by which tamoxifen manifests SERM activity is not yet known. Evidence presented in this study suggests that the tamoxifen-bound receptor exposes a binding site that is occupied by a coactivating protein not primarily used by the estradiol-activated receptor. The α II peptide, which interacts with both estradiol- and tamoxifen-bound receptors, inhibits the partial agonist activity of tamoxifen efficiently, while minimally affecting estradiol-mediated transcription. This result suggests that this site, although crucial for tamoxifen-mediated transcription, is dispensable for estrogen action. In addition, the ability of α / β III and α / β V to bind tamoxifen-specific surfaces and inhibit tamoxifen-mediated partial agonist activity suggests that these peptides may potentially recognize a protein contact site on ER that is critical for this activity. In this regard, we can

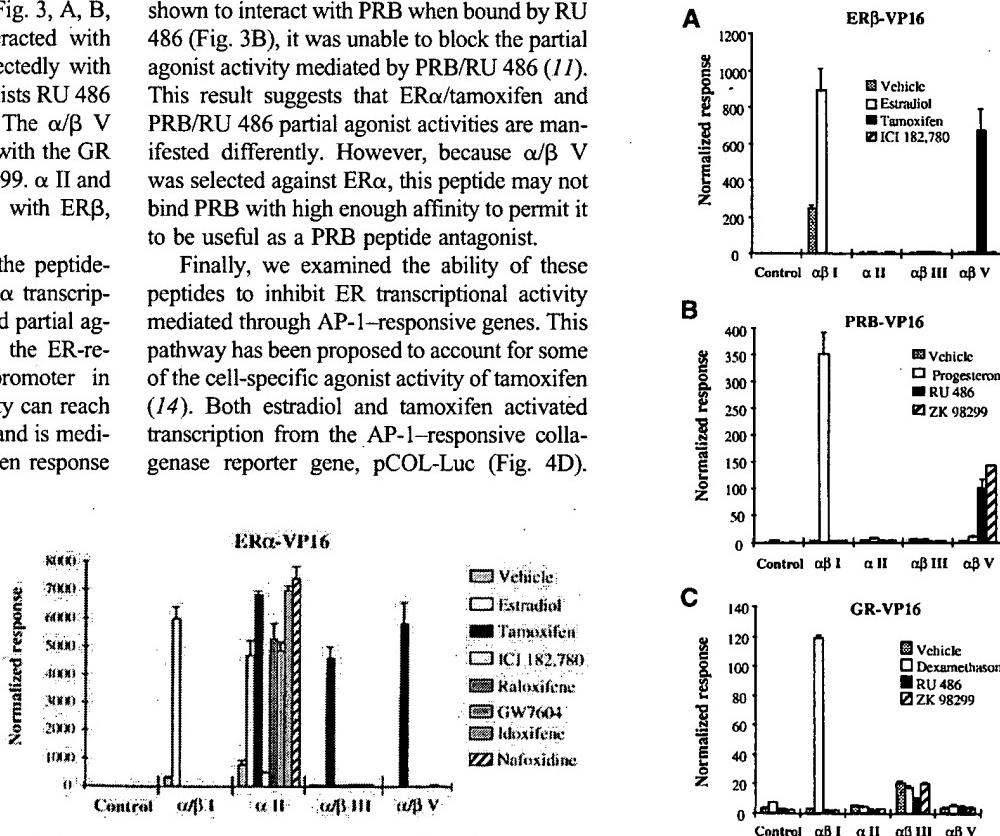


Fig. 2. ER α -peptide interactions in mammalian cells. The coding sequence of a peptide representative from each class identified was fused to the DBD of the yeast transcription factor Gal4. HepG2 cells were transiently transfected with expression vectors for ER α -VP16 and the peptide-Gal4 fusion proteins. In addition, a luciferase reporter construct under the control of five copies of a Gal4 upstream enhancer element was also transfected along with a pCMV- β -galactosidase (β -Gal) vector to normalize for transfection efficiency. Transfection of the Gal4 DBD alone is included as control. Cells were then treated with various ligands (100 nM) as indicated and assayed for luciferase and β -Gal activity. Normalized response was obtained by dividing the luciferase activity by the β -Gal activity. Transfections were performed in triplicate, and error bars represent standard error of the mean (SEM). Triplicate transfections contained 1000 ng of ER α -VP16, 1000 ng of 5X Gal4-tata-Luc, 1000 ng of peptide-Gal4 fusion construct, and 100 ng of pCMV- β -Gal (70).

Fig. 3. Specificity of nuclear receptor-peptide interactions. Two-hybrid experiments were performed as in Fig. 2 between peptide-Gal4 fusion proteins and either (A) ER β -VP16, (B) PRB-VP16, or (C) GR-VP16 (15). RU 486 and ZK 98299 are pan-antagonists of PRB and GR.

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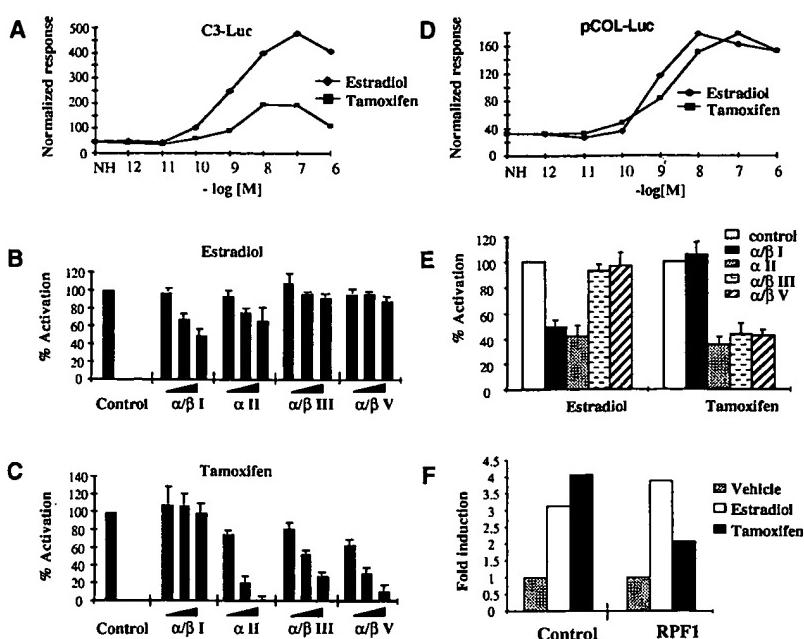


Fig. 4. Disruption of ER α -mediated transcriptional activity. (A) HepG2 cells were transfected with the estrogen-responsive C3-Luc reporter gene (12) along with expression vectors for ER α (16) and β -Gal and normalized as in Fig. 2. Cells were induced with either estradiol or tamoxifen as indicated and analyzed for luciferase and β -Gal activity. NH, no hormone. (B) HepG2 cells were transfected as in (A) except that expression vectors for peptide-Gal4 fusions were included as indicated. Control represents the transcriptional activity of estradiol (10 nM)-activated ER α in the presence of the Gal4 DBD alone and is set at 100% activity. Increasing amounts of input plasmid for each Gal4-peptide fusion are also shown (Δ) with the resulting transcriptional activity presented as percentage of activation of control. Data are averaged from three independent experiments (each performed in triplicate) with error bars representing SEM. Triplicate transfections contained 1000 ng of C3-Luc, 1000 ng of ER α expression vector, 100 ng of pCMV- β -Gal, and either 100, 500, or 1000 ng of peptide-Gal4 fusion construct. (C) Same as in (B) except that 4-OH tamoxifen (10 nM) was used to activate the receptor. (D) HepG2 cells were transfected with the AP-1-responsive collagenase reporter gene construct (pCOL-Luc) (12) and expression vectors for ER α and β -Gal. Cells were then induced with either estradiol or tamoxifen as indicated. (E) Same as (D), except that peptide-Gal4 fusion constructs were also transfected as indicated. Control represents the transcriptional activity of either estradiol- or tamoxifen (100 nM)-activated ER in the presence of the Gal4 DBD alone and is set at 100% activity. The transcriptional activity of estradiol and tamoxifen is shown in the presence of each Gal4-peptide fusion with the resulting transcriptional activity presented as percentage of activation of control. Triplicate transfections contained 1000 ng of pCOL-Luc, 1000 ng of ER α expression vector, 1000 ng of peptide-Gal4 fusion construct, and 100 ng of pCMV- β -Gal. Data are presented as in (B) and (C). (F) HeLa cells were transfected with the 1X-ERE-tata-Luc reporter gene along with expression vectors for ER α , β -Gal, and either RPF1 (pCDNA3-RPF1) or control vector [pCDNA3 (Invitrogen, Carlsbad, CA)]. Cells were induced with ligand (10 nM) as indicated. Data are presented as fold induction, which represents the ratio of ligand induced versus vehicle for each transfection.

demonstrate that, similar to α/β V, overexpression of RPF1 specifically represses tamoxifen-mediated partial agonist activity (Fig. 4F). However, the physiological importance of this activity remains to be determined. In summary, we have identified a series of peptide antagonists of ER α and hence validated additional target sites other than the ligand-binding pocket for drug discovery.

References and Notes

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- Phage display was performed as described [L. A. Paige *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3999 (1999)]. Immulon 4 96-well plates (Dynex Technologies, Chantilly, VA) were coated with streptavidin in NaHCO₃ buffer (pH 8.5) at 4°C for about 18 hours. Wells were blocked with bovine serum albumin (BSA) and then washed with TBST [10 mM tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20], and 2 pmol of biotinylated vitellogenin ERE was then added per well. Plates were washed with TBST, 3 pmol of baculovirus-purified ER α or ER β (Pan Vera, Madison, WI) was then added, and plates were incubated at room temperature for 1 hour. Hormone was then added (1 μ M) along with phage library (containing about 1.5 \times 10¹⁰ phage) in TBST and incubated at room temperature for 1 hour. Nonbinding phage were removed by washing with TBST. Bound phage were eluted in prewarmed (50°C) 50 mM glycine-HCl (pH 2.0). Eluent was neutralized by the addition of 200 mM Na₂HPO₄ (pH 8.5), and phage were amplified in *Escherichia coli* (DH5 α F'). Affinity selection was repeated three times, and individual phage were isolated from either the second or third round of amplification. Peptide sequences were then deduced by DNA sequencing.
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- TRF assays were performed at room temperature as follows: Costar (Cambridge, MA) high-binding 384-well plates were coated with streptavidin in 0.1 M sodium bicarbonate and blocked with BSA. Twenty microliters of biotinylated ERE (100 nM in TBST) was added to each well. After a 1-hour incubation, biotin (50 μ M in TBST) was added to block any remaining binding sites. The plates were washed, and 20 μ l of ER α (100 nM in TBST) was added to each well. After a 1-hour incubation, the plates were washed, and 5 μ l of 5 μ M 4-OH tamoxifen was added to each well followed by 15 μ l of solution containing the peptides conjugated to unlabeled streptavidin (prepared as described below) at a range of concentrations (from 1.67 μ M in twofold dilutions). After a 30-min incubation with the 4-OH tamoxifen and conjugate, 5 μ l of 400 nM europium-labeled streptavidin (Wallac, Gaithersburg, MD)-biotinylated peptide conjugate (prepared as described below) was added and incubated for 1 hour. The plates were then washed, and the europium enhancement solution was added. Fluorescent readings were obtained with a POLARstar fluorimeter (BMG Lab Technologies, Durham, NC) with a <400-nm excitation filter and a 620-nm emission filter. The streptavidin-biotinylated peptide conjugates were prepared by adding 4 pmol of biotinylated peptide per picomole of streptavidin. After incubation on ice for 30 min, the remaining biotin-binding sites were blocked with biotin before addition to the ER-coated plate.
- HepG2 cells were maintained in modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies). Transfections were performed as described [J. D. Norris *et al.*, *J. Biol. Chem.* **270**, 22777 (1995)]. pCMV- β -Gal and 5X GAL4-tata-Luc were described previously [B. L. Wagner, J. D. Norris, T. A. Knotts, N. L. Weigel, D. P. McDonnell, *Mol. Cell. Biol.* **18**, 1369 (1998)]. Gal4 DBD-peptide fusions were created as follows: Peptide-coding sequences were excised from mBAX vector with Xba I-Xba I and subcloned into pMV vector (Clontech, Palo Alto, CA) with a linker sequence to generate Sal I and Xba I sites for cloning. ER α -VP16 was generated by polymerase chain reaction (PCR) of human ER α -cDNA containing Eco RI sites flanking both 5' and 3' termini. The resultant PCR product was then subcloned into pVP16 (Clontech). All PCR products were sequenced to ensure the fidelity of the resultant construct. 17 β -estradiol, 4-hydroxy-tamoxifen, and nafoxidine were purchased from Sigma.
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- ER β -VP16 was generated by PCR of ER β cDNA, and the resultant product was cloned into pVP16. Dexamehtasone and progesterone were purchased from Sigma.
- ER α expression vector pRST7-ER α is reported elsewhere [S. L. Dana, P. A. Hoener, D. L. Wheeler, C. L. Lawrence, D. P. McDonnell, *Mol. Endocrinol.* **8**, 1193 (1994)].
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